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ELECTROLYSIS-MEDIATED IRREVERSIBLE INACTIVATION OF LIPOXYGENASE DIRECTED TOWARD ELECTROAFFINITY LABELLING

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SUMMARY: Irreversible inhibition of soybean lipoxygenase-1 (SL-1) was accomplished via a controlled potential oxidative electrolysis of 1,5-dihydroxynaphthalene (1,5-DHN) at +0.8 V vs SCE. The inactivation of SL-1 with this known inhibitor was greatly enhanced under these electrolytic conditions to which the enzyme itself was stable. Electrolyses were run at 0°C in a 0.05 M phosphate buffer, pH 7.0, using graphite cloth electrodes. The rate of inactivation was observed to be limited by and dependent on the anodic oxidation of 1,5-DHN. The non-oxidizable (at this potential) inhibitor indomethacin was shown to protect the enzyme from irreversible inactivation, however, an external nucleophile (2-mercaptoethanol) had little effect. These initial studies support the capability of such electrochemical methods for the site-specific covalent modification (affinity labelling) of lipoxygenase, and perhaps other enzymes.

A large number of electrochemical techniques have been used to study various enzymes and other biological macromolecules. However, there have been no reported attempts to electrochemically mediate the site-specific covalent attachment of an electroactive ligand to any protein. The radiolabelling of yeast cell membranes by *in situ* oxidative electrolysis of $Na^{125}I$ showed the feasibility of non-selective modification using such methods (1). Site-selective covalent binding mediated by electrolyses of enzyme substrate analogs or inhibitors represents a special category of the general "affinity labelling" approach (2), which is in some ways analogous to photo-affinity labelling (3), where reactive intermediates are generated *in situ* by photolytic techniques.

Soybean lipoxygenase-1 (linoleate oxidoreductase EC 1.13.11.12) was selected for these initial trials due to its ready availability and its observed sensitiv-

ABBREVIATIONS: SL-1, soybean lipoxygenase-1 (linoleate oxidoreductase EC 1.13.11.12); 1,5-DHN, 1,5-dihydroxynaphthalene; 1,3-DHN, 1,3-dihydroxynaphthalene; SCE, saturated calomel electrode.

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ity to various potentially electroactive inhibitors (4). The inhibitor 1,5-dihydroxynaphthalene (1,5-DHN) was selected due to its known enzymatic inhibitory properties and its propensity to form a highly-reactive quinonoid species upon oxidation (5,6). These studies paralleled our investigation of quinonoid derivatives of known inhibitors of the enzyme cycloxygenase, as previously reported (7).

Although the exact mechanism of hydroperoxidation catalyzed by SL-1 has not been established, a non-heme iron and a residue are apparently involved (8). A number of acetylenic mechanism-based irreversible inhibitors of SL-1 have been reported (9,10) and alpha-bromostearic acid has been claimed to be an inately-reactive affinity label for this enzyme (8). No electrolytic inactivation of this enzyme has been previously reported.

MATERIALS AND METHODS

Arachidonic acid, indomethacin, and soybean lipoxygenase (165,000 U/mg) were obtained from Sigma Chemical Company. 1,5-Dihydroxynaphthalene, 1,3-dihydroxynaphthanlene, hydroquinone, juglone, and 2-mercaptoethanol were obtained from Aldrich Chemical Company. 1,5-DHN was recrystallized from acetonitrile prior to use. The lipoxygenase was dissolved (8 μg/ml) in 0.05 M phosphate buffer, pH 7.0. All inhibitors were dissolved in ethanol as a co-solvent (50 mM concentration) and stored at 0°C under nitrogen. Previous reports of the solution stability of this enzyme and sensitivity to ethanol were taken into account (11-13).

Cyclic Voltammetry: Oxidation potentials were determined utilizing a Princeton Applied Research (PAR) Model 173 potentiostat in conjunction with a PAR Model 175 Universal programmer. Measurements were performed in 10% aqueous acetonitrile (1.5 mM concentration with 0.1 M $LiClO_4$) utilizing a freshly polished glassy carbon electrode with a saturated calomel electrode as a reference. The oxidation curve of 1,5-DHN indicated one peak (+0.7V) and 1,3-DHN two peaks (+0.7V and +1.1V).

Electrochemical oxidations: Controlled potential electrolyses (+0.80 V vs SCE) with Bioanalytical Systems SP-2 potentiostat were carried out at 0°C in 0.05 M potassium phosphate buffer, pH 7.0 using a four-neck chamber with one neck (cathode) separated from the enzyme solution by a medium porosity glass frit to which an agar gel (1.0 N NaCl) had been applied. Electrodes (0.6 x 1.0 x 2.5 cm) were cut from graphite felt (0.25 in No.GSGC-2; Carborundum Corp. Sanborn, NY). The SCE reference electrode was obtained from Aldrich Chemical Co.

Assay of Lipoxygenase Activity: Enzyme activity was determined polarographically in a manner previously described (14) utilizing a Gilson Oxygraph Model K-1C and a Clark oxygen electrode. The reaction cell (2.0 ml) was water-jacketed for temperature control (22 ±1°C) and equipped with a small magnetic stirring device. The lipoxygenase reaction was initiated by the addition of arachidonic acid (80 μg in 20 μl of ethanol). Initial velocities were obtained from the slope of the oxygen concentration vs time curve.

UV measurements and dialysis: Ultraviolet spectra were recorded utilizing a Beckman DU-8 Spectrophotometer. Dialysis of inhibited enzyme samples and unin-

hibited controls were carried out over a 23-hour period with a single buffer change. Dialysis tubing was prepared by boiling for 30 minutes in a 0.5 M EDTA solution and followed by similar treatment (six times) with deionized water.

RESULTS AND DISCUSSION

An investigation of the effects of inhibitor (1,5-DHN) concentration vs time period of electrolysis (Figure 1) revealed that the inactivation process was first order with respect to active enzyme remaining. No inactivation was observed in the absence of inhibitor. For each concentration of inhibitor utilized (50-300 μM 1,5-DHN) pseudo-first order kinetics were observed and inactivation half-lives ($t_{1/2}$ could be determined. A plot of $t_{1/2}$ vs the reciprocal of inhibitor concentration (Figure 2) allowed the determination of the inhibition constant (K_i = 833 μM) and the first-order rate constant (k_{inact} = 5.2 x 10^{-3} sec^{-1}). The linearity of this plot and the observed positive y-intercept support the forma-

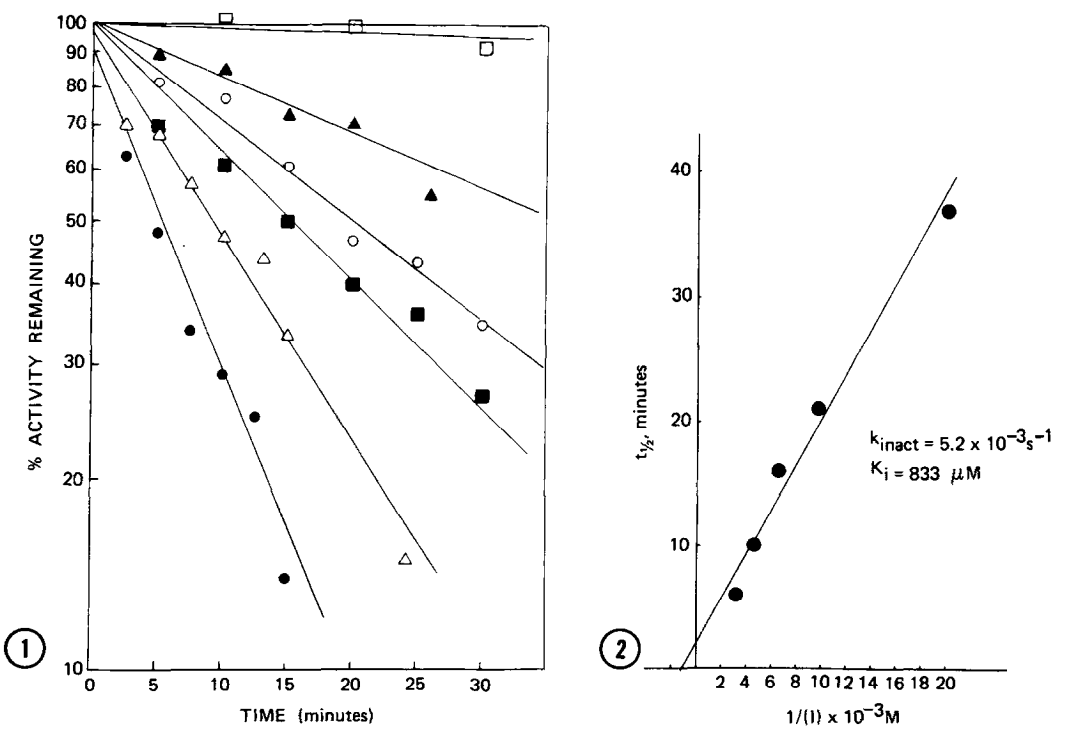
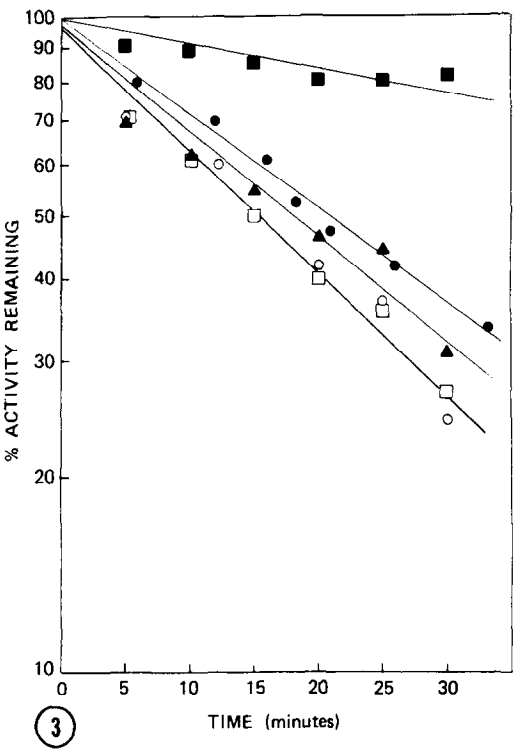


Figure 1. Inhibition of soybean lipoxygenase by electrolysis in the presence of 1,5-dihydroxynaphthalene (1,5-DHN): □ (no inhibitor present); ▲ (50 μM concentration 1,5-DHN); ○ (100 μM 1,5-DHN); ■ (150 μM 1,5-DHN); △ (225 μM 1,5-DHN); ● (300 μM 1,5-DHN).

Figure 2. Plot of inactivation half-life ($t_{1/2}$, min) vs the reciprocal of inhibitor concentration, [I].



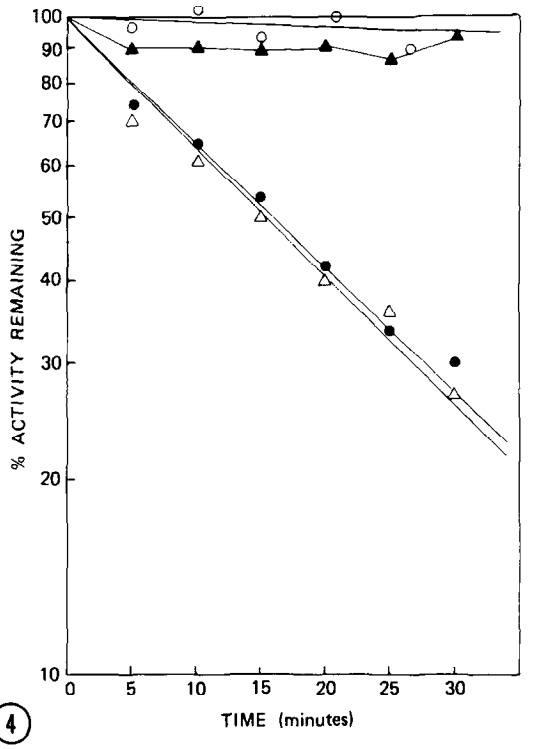


Figure 3. Observation of the effects of (a) an external nucleophile (2-mercaptoethanol) at two concentrations (○ 150 μM; ▲ 300 μM); (b) a nonoxidizable competitive inhibitor (indomethacin, ● 500 μM) and (c) ■ electrolysis of 1,5-DHN (300 μM) prior to addition of the enzyme.

Figure 4. Inhibitory effects of electrolyzed hydroquinone (○ 150 μM); 1,3-dihydroxynaphthalene (● 150 μM); and non-electrolyzed juglone (▲ 150 μM) as compared to 1,5-DHN (△ 150 μM).

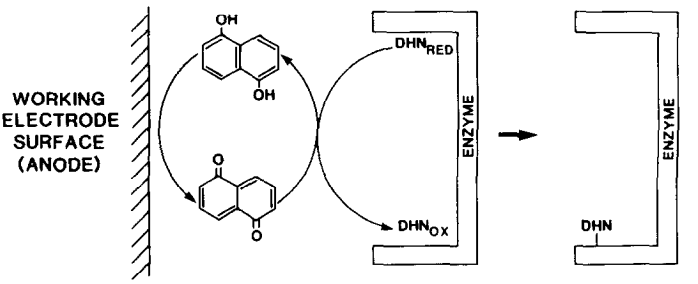
tion of a reversible enzyme-inhibitor complex prior to inactivation. It was also observed in a separate experiment (Figure 3) that the addition of an electrochemically-oxidized solution of 1,5-DHN (300 μM) to a non-electrolyzed enzyme solution did not lead to enzyme inactivation, thus revealing the necessity of proximity between enzyme and inhibitor as discussed later.

Attempts to ascertain the nature of the inactivating species in this electrochemical process are illustrated in Figures 3 and 4. In the first instance (Figure 3) it is observed that the external nucleophile, 2-mercaptoethanol (up to 300 μM) has little influence on the inactivation process, thus arguing against the formation of a freely-diffusible electrophilic quinone which would be expected to react with this sulfhydryl reagent. The modicum of protection to irreversible inactivation provided by the competitive inhibitor indomethacin (500 μM), which

by itself does not inhibit lipoxygenase under these conditions, suggest that any oxidized species arising from 1,5-DHN is formed in close proximity to the site of inactivation and is not easily displaced by a competing inhibitor.

Further insight into the potential identity of the irreversible inhibitor derived from 1,5-DHN by electrolytic oxidation was gained by experiments shown in Figure 4. Juglone, which has been identified as a product of anodic oxidation of 1,5-DHN in 2N $HClO_4$ (5), was observed to have little direct effect upon lipoxygenase activity (150 μM concentration). Likewise, hydroquinone, a less lipophilic molecule which is oxidizable at the potential utilized, showed no inhibition of lipoxygenase activity upon electrolysis, presumably due to its lack of affinity for the enzyme's inhibitory site. Somewhat surprisingly, the isomeric 1,3-dihydroxynaphthalene (1,3-DHN) upon electrolysis showed an inhibitory effect which is almost identical to that of 1,5-DHN. This inhibition by 1,3-DHN along with the lack of inhibition observed with juglone suggest that the inactivating species in an intermediate oxidation product (e.g. semiquinone or phenoxy radical) rather than a quinone product which cannot be formed by the 1,3-DHN isomer. Further experimentation will be necessary to identify the inactivating species in this irreversible process.

Several experiments have been performed to determine the potential reversibility of this observed electrochemical inactivation and to characterize modification of the enzyme's structure in the inactivation process. Reversibility of inactivation by dialysis is complicated by the instability of SL-1 to long term dialysis under dilute conditions (12). In a typical experiment, lipoxygenase (14 μg/ml) which had been treated with 225 μM 1,5-DHN and electrochemically oxidized for 30 minutes (40% loss of enzyme activity), was dialyzed over a period of 23 hours. During this time period an untreated enzyme solution lost approximately 70% lipoxygenase activity. However, a comparison of relative enzyme activity after dialysis showed the same additional 40% loss due to the electrochemical process. An ultraviolet absorption comparison of 1,5-DHN treated and and untreated SL-1 dialyzed samples revealed incorporation of the strong DHN chromaphore (multiple λ_{max} 280-330 nm) into the protein structure.



Scheme 1. Proposed mechanism for irreversible electrolytic inactivation of soybean lipoxygenase.

CONCLUSIONS

Based upon the preceding observations we can conclude that *in situ* anodic oxidation of 1,5-DHN in the presence of soybean lipoxygenase leads to irreversible enzyme inactivation as a result of covalent modification of the enzyme. These observations support the use of such techniques for selective chemical modification of lipoxygenase and may serve as precedence for the development of electroaffinity labelling procedures for other proteins as well. A reasonable scheme (Scheme 1) is suggested to account for the observed kinetics of this inactivation process and to explain the relative invulnerability of this process to competitive inhibition and the effects of an external nucleophile. This scheme suggests the intermediacy of 1,5-DHN oxidation products as redox couplers which oxidize enzyme bound 1,5-DHN, however, electron transfer directly between the anode and protein structure might also serve this intermediary role (14). A further exploration of this labelling technique will be pursued with structurally-modified inhibitors and alternative enzyme systems.

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